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Oncomiracidial Behavior of *Heterobothrium okamotoi* (Monogenea: Diclidophoridae)

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ABSTRACT—We conducted a series of experiments to understand the behavioral characteristics of oncomiracidium of the monogenean *Heterobothrium okamotoi* and compared them with those of two other monogenean species, *Heteraxine heterocerca* and *Neobenedenia girellae*. Behavior of *H. okamotoi* oncomiracidium consisted of two alternate phases: a swimming phase with strong ciliary beatings and a stationary phase with ciliary beatings too weak to generate any movements. The duration of one swimming-stationary cycle considerably varied among individuals but tended to increase with larval age. During the 5-day monitoring, length of the swimming phase decreased while that of the stationary phase tended to increase as the larva became older. The locomotion patterns differed significantly among individuals, but as a whole, larvae showed random horizontal movement and no phototactic reaction. The oncomiracidium tended to move downward because it was heavier than seawater and also swam downward at the start of the swimming phase because its anterior part pointed downward when the larva was not swimming. Those behavioral characteristics of larval *H. okamotoi* are likely to have evolved in order to maximize the chance of encountering its benthic fish host, tiger puffer *Takifugu rubripes*.

Key words: *Heterobothrium okamotoi*, oncomiracidium, behavior, Monogenea, *Takifugu rubripes*

Culture of tiger puffer *Takifugu rubripes* using floating net cages has become increasingly popular in Japanese coastal waters since the 1980s. Concurrently, infection by the monogenean *Heterobothrium okamotoi* has became a major problem in the puffer culture industry. This is mainly because eggs of *H. okamotoi* entangle with the cage net and hatched larvae (oncomiracidia) can gain easy access to the host (Ogawa, 2002). Combining this with the high culturing density, farmed tiger puffer often suffers from heavy infection which results in slowed growth and increased mortality. Formalin bathing of infected fish has traditionally been practiced as an effective control measure for this monogenean infection. However, use of formalin was strictly banned since 2003 due to increasing concerns about food safety and environmental protection. Chemotherapy using hydrogen peroxide or febantel, a benzimidazole anthelmintic, can be alternative options. However, these methods are not an ultimate solution because such chemical treatments, though effective, cannot eradicate the parasite from the culturing area. Therefore, it is important to prevent the parasite from entering and multiplying within the culturing water. Combination of chemotherapy and prevention would provide a more effective and less labour intensive control method.

An essential step to develop the preventive measure is to understand the general biology of the target pathogen. In this context, biological aspects of *H. okamotoi,* especially the reproductive characteristics and biology of oncomiracidium, have previously been studied. These include studies on egg production (Ogawa, 1997; Yamabata *et al*., 2004), egg laying (Ogawa *et al*., 2005), egg hatching, morphology and life span of oncomiracidium (Ogawa, 1998), attachment process (Chigasaki *et al*., 2000; Yoshinaga *et al*., 2006) and host recognition (Ohhashi *et al*., 2007). However, very little is known about the behavioral biology of the oncomiracidium and how it disperses in the water. Such information provides important knowledge to develop a preventive method (Ogawa, 2002). In the present study, we describe the behavioral characteristics of *H. okamotoi* oncomiracidium by series of laboratory experiments. In addition, to understand the association between the larval behavior and ecology of its host, behavioral characteristics of *H. okamotoi* were compared with two other species of monogeneans important in aquaculture.

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Materials and Methods

Animals

Eggs of *Heterobothrium okamotoi, Heteraxine heterocerca* (Heteraxinidae) and *Neobenedenia girellae* (Capsalidae) were collected from the tanks rearing infected tiger puffer *Takifugu rubripes*, yellowtail *Seriola quinqueradiata* or olive flounder *Paralichthys olivaceus*, respectively. Eggs of *H. okamotoi* and *H. heterocerca* were also obtained by incubating adult worms in a Petri dish with natural sea water at room temperature. Collected eggs were thoroughly rinsed and incubated in filtered natural sea water. In order to obtain freshly hatched larvae on the day of experimental trial, timing of hatching was controlled by setting incubation temperatures at 20 or 15°C. Occasional mechanical stimuli were given during the incubation to induce hatching. Hatched larvae were collected by a 20 μm mesh, gently washed and placed in a separate container with fresh natural sea water. Larval collection was conducted daily so that the oncomiracidia in a container were approximately at the same age (< 24 h). The larval density in a suspension was estimated by counting the numbers of oncomiracidia in 1 mL sample. Hatched larvae were kept at the incubation temperature until used.

Physical characteristics of oncomiracidium

Body positioning and free sinking velocity of the oncomiracidia were assessed in an acrylic chamber (50 × 100 × 5 mm, all dimensions here and hereafter are of inner size) and in a glass tube (6 mm in diameter, 900 mm in length), respectively. Freshly hatched larvae were first treated in $NiCl_2$-added artificial sea water (consisting of 450 mM NaCl, 9 mM KCl, 40 mM $MgCl_2$, 10 mM $CaCl_2$ and 2 mM HNa_2O_4P; salinity, 32.16 ppt) for 20 min to inhibit ciliary beatings (Naitoh and Kaneko, 1973) and then placed in an acrylic chamber to video monitor its positioning. To measure the sinking speed, a $NiCl_2$ treated oncomiracidium was placed in a glass tube filled with artificial sea water and its distance from the releasing point was measured every 10 min.

Swimming pattern of oncomiracidium

1) Video analysis

Behavior of *H. okamotoi* oncomiracidia was video monitored. Five larvae were placed together in a cylindrical glass chamber (20 mm in diameter; 1.0 mm in depth) and recorded for 15 min from above using a digital CCD camera. A fiber optic illuminator (Hayashi Watch-works, Co., Ltd., LA-50UE) was used as a light source and the water temperature was 21 ± 1°C. Our preliminary observations showed that swimming behavior consisted of two alternate phases: swimming and stationary phases. During the swimming phase, the larva shows vigorous ciliary beating and moves in random trajectory. During the stationary phase, ciliary beating becomes too weak to generate any forward movement and the larva stays at the same position.

Recorded video images were captured onto a computer (30 frames per s) and the swimming trajectory of an individual larva was traced for five consecutive swimming-stationary cycles. Any swimming behavior distracted by touching the edge of the chamber was excluded from the analysis. In addition, vertical movement of the oncomiracidia was video monitored in the acrylic chamber described above.

2) Visual observation

Temporal changes in swimming behavior of *H. okamotoi* oncomiracidia associated with time post-hatch were assessed. Durations of each swimming and stationary phase were measured everyday from immediately after hatch to 5 days post hatch. A single oncomiracidium was placed in a transparent acrylic cylinder (18 mm in diameter, 900 mm in length) and its behavior was visually observed with the aid of the fiber optic light. Duration of each phase was measured by a stopwatch for 5 min or in the period of 50 swimming cycles whichever came first. The observations were made for ten oncomiracidia each day.

In contrast to *H. okamotoi*, oncomiracidia of *H. heterocerca* kept swimming forward all the time. Their swimming pattern consisted of two alternate phases: fast and slow swimming phases. The fast swimming phase in *H. heterocerca* corresponds to the swimming phase in *H. okamotoi*. In the slow swimming phase, their swimming speed decreased clearly, but continuously moved around. This phase is different from the stationary phase of *H. okamotoi* which stays at one position. Durations of each phase were measured using ten freshly hatched oncomiracidia using the same methods used for *H. okamotoi*.

Dispersal and taxis of oncomiracidium

Horizontal dispersion and phototaxis of *H. okamotoi* oncomiracidia were investigated. Artificial sea water containing 100 freshly hatched oncomiracidia was added into the a long clear acrylic pipe (18 mm in diameter, 500 mm in length; calculated volume 127.2 mL) and the sealed pipe was laid horizontally. The entire pipe was covered with a black vinyl sheet except for the 3 cm area from one end. The uncovered part of the pipe was illuminated by the fiber optic light (light intensity not measured) set at vertical to the pipe for 30 min, and then the pipe was gently set to the vertical position. Numbers of oncomiracidia in every 10 cm (25.4 mL) water column were counted by siphoning out the water from the top. Light illumination was turned off during the collection process and the process was completed within approximately 3 min. The experiment was also performed with *N. girellae* oncomiracidia,

which acts as positive control because it is known to have strong positive phototaxis (Ishida *et al*., 2007). To compare the distribution of larval *H. okamotoi* in darkness, the experiment was repeated using the pipe entirely covered with the black vinyl sheet and without any light illumination (negative control). All experiments, except for the negative control, were duplicated.

Horizontal dispersal of *H. okamotoi* was investigated in a large glass dish (220 mm in diameter, 46 mm in depth) filled with natural sea water to 6 mm in depth. Approximately 100 freshly hatched oncomiracidia stained with carboxyfluorescein succinimidyl ester (CFSE; Chigasaki *et al*., 2000) were placed in the middle of the dish which was set on the Lumino image analyzer (Fujifilm, LAS-1000 plus). Larval distribution was recorded from above by a digital camera for a total of 60 min; a 5 min interval for the first 30 min and 10 min interval thereafter. The images were analysed on a computer to determine the number of oncomiracidia within every 2 cm radius area from the releasing point.

Vertical dispersion of *H. okamotoi* oncomiracidia was investigated using a similar setup as described above. One hundred oncomiracidia were gently added from the top of a vertical clear acrylic pipe (90 mm in working length) filled with natural sea water. The pipe was left to stand and the numbers of oncomiracidia in every 10 cm water column were counted after 1, 3 and 24 h by the same procedure described above. This was designated as "top-released group". The same experiment was repeated except that this time the pipe was turned upside down immediately after larvae were added. This was designated as "bottom-released group". Both experiments were repeated using the oncomiracidia at different ages (immediately after hatch, 1 day- or 2 days-old) and the trials were duplicated.

Infection experiment

During the experiment to determine the vertical dispersion of the larval *H. okamotoi*, we noted that small portions of oncomiracidia remained in the top water column while most larvae were found at the bottom. To compare the infectivity of these "top" and "bottom" larvae, an infection experiment was conducted. Three hours old oncomiracidia were placed in a glass cylinder (28 mm in diameter, 250 mm in height) and left undisturbed for 24 h. A total number of 136 larvae were found within the 2 cm water colum from the surface. These larvae were stained with CFSE and added to a bucket containing five young tiger puffer (mean body length 9.3 cm, ca. 27 larvae per fish) in 5 L natural sea water. The same numbers of larvae were collected from 2 cm water column at the bottom of the cylinder and were used to infect the fish in the same manner. After 3 h of exposure at 20°C, fish were dissected and numbers of larvae attached onto body surface and gills were counted using a florescence microscope.

Statistical analyses

Data were tested for normality using Shapiro-Wilkes test. For the statistical analyses involving durations of swimming phase, mean of each phase was calculated for individuals and compared between different larval ages using Kruskal-Wallis. For comparison of the parasite intensity, Wilcoxon Rank Sum test was used. Chi-square test was applied for the analyses of the larval distribution in the pipes and infection rates of oncomiracidia. The averages of duplicated trials were used for analyses in phototaxis and horizontal distribution experiments. Analyses were performed using JMP statistic software version 7 (SAS Institute).

Results

Physical characteristics of oncomiracidium

The mean sinking velocity of the oncomiracidia was 8.3 ± 0.5 cm/min (n = 10). Nearly all of the $NiCl_2$ treated larvae were found tilting their anterior part downward.

Swimming pattern of oncomiracidium

Video analyses showed that *H. okamotoi* oncomiracidia repeated alternate swimming and stationary phases. In close observations, the oncomiracidia moved forward, following a spiral path with vigorous ciliary beatings during the swimming phase. In the stationary phase, beatings of cilia were too weak to generate forward movement. There was no obvious directional preference in horizontal axis and no movement pattern preference was observed as individual larva showed great variation in their movements (Fig. 1). Some larvae showed continuous circular movement while others followed apparently random trajectory. Visual observations also revealed that the duration of the swimming and stationary phases varied considera-

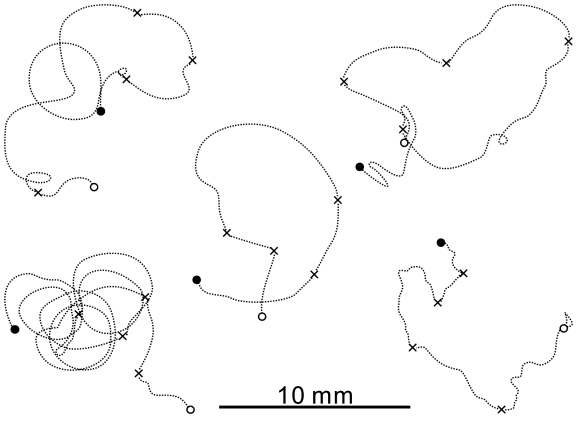

Fig. 1. Examples of swimming pattern of *Heterobothrium okamotoi* oncomiracidium in a container (five individuals during five swimming-stationary cycles). Open circle, close circle and X represent starting point, end point and stationary point, respectively.

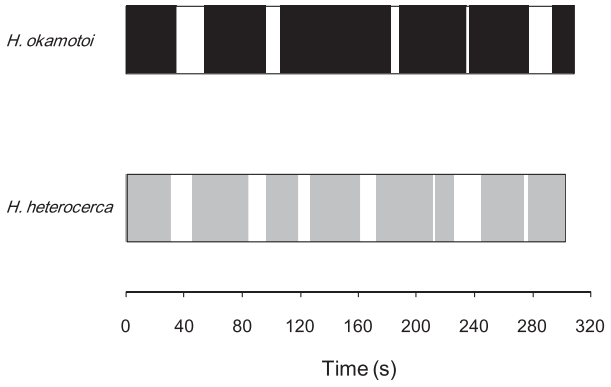

Fig. 2. Examples of swimming pattern of *Heterobothrium okamotoi* oncomiracidium (top) and *Heteraxine heterocerca* oncomiracidium (bottom). Black and white bars for *H. okamotoi* represent stationary and swimming phases, respectively. Gray and white bar for *H. heterocerca* represent fast and slow swimming phases, respectively.

bly not only among individuals but also within a single larva. For example, the duration of one swimming and stationary phase of a freshly hatched oncomiracidium ranged from 2.1 to 18.8 (mean 10.2 ± 6.2, SD here and elsewhere) s and from 15.2 to 75.9 (mean 42.8 ± 19.6) s, respectively. This particular individual showed six swimming-stationary cycles in 317 s observation (Fig. 2). In general, the stationary phase was longer than the swimming phase. Within the acrylic chamber, most oncomiracidia remained at the bottom and showed very little vertical movement. Some swam upward but slowly sank as they stopped swimming. Small proportion of larvae swam toward the surface and remained in the upper water column.

Oncomiracidia of *H. heterocerca* kept swimming forward all the time with two alternate speeds as previously described. For comparison, particular swimming patterns of one freshly hatched *H. heterocerca* and *H. okamotoi* larvae are plotted on Fig. 2. The ranges of the fast and slow swimming phase of this particular *H. heterocerca* larva during eight swimming cycles in 305 s were 1.2–19.2 (mean 8.8 ± 6.4) s and 13.0–39.4 (mean 29.3 ± 9.0) s, respectively. The overall mean duration of one fast-slow swimming cycle of 10 freshly hatched *H. heterocerca* was 72.1 ± 45.8 s (range 28.2–158.0 s).

Temporal change in duration of swimming and stationary phases of all examined *H. okamotoi* larvae is plotted on Fig. 3. For freshly hatched larvae, the mean durations of one swimming and stationary phase were 7.1 ± 6.0 s and 14.8 ± 11.7 s, respectively. There was a general tendency that the duration of the swimming phase decreased whereas that of stationary phase increased as larvae became older. The age of larvae had a significant effect on the duration of each phase (Kruskal-Wallis, swimming; $p = 0.018$, stationary; $p = 0.002$). Duration of one swimming-stationary cycle also changed with larval age ($F_{5,54} = 2.49$, $p < 0.042$, Fig. 4). In comparison with a swimming cycle of freshly hatched *H. heterocerca*, one swimming-stationary cycle of *H. okamotoi* was significantly shorter ($F_{1,18} = 10.79$, $p < 0.0041$).

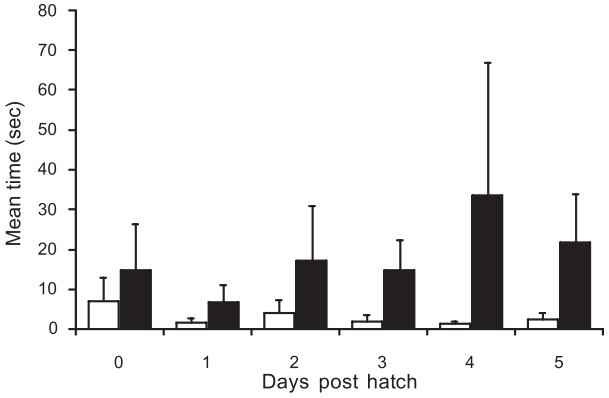

Fig. 3. Age-dependent changes in the swimming rhythm of *Heterobothrium okamotoi* oncomiracidium. Open and closed columns represent swimming and stationary phases, respectively. Data are expressed as mean + SD.

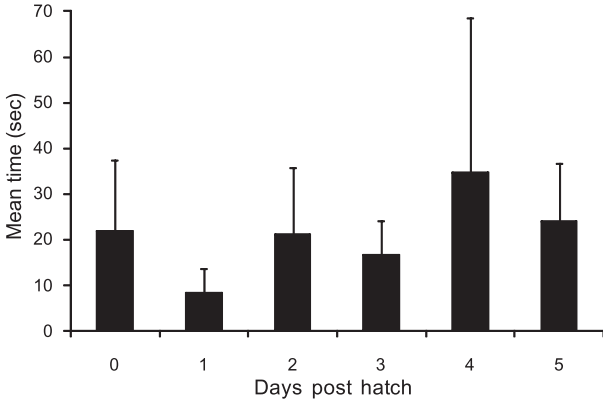

Fig. 4. Changes in duration of the swimming-stationary cycle in the 5 days observation period. Data are expressed as mean + SD.

Dispersal and taxis

The light illumination did not affect the horizontal distribution of *H. okamotoi* (Chi-quare, $p = 0.8584$, Fig. 5). Regardless of the illumination, larvae were almost evenly distributed within the pipe and the proportions of larvae found in each 10 cm water column were 15.7% to 24.6% (range). Contrastingly, over 80% of *N. girellae* larvae were found in the water column nearest to the light source which clearly indicates their positive phototaxis (Chi-square, $p < 0.0001$, Fig. 5).

In the experiment on the vertical dispersion, the majority (over 50% in most cases) of top released and bottom released larvae were found at the bottom 10 cm of the pipe (Fig. 6). The proportion of larvae found in

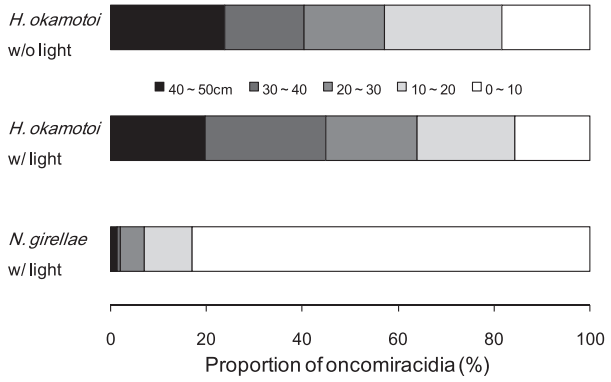

Fig. 5. Distance from the illuminated water column for *Heterobothrium okamotoi* oncomiracidium (top figure) and in control (dark; middle figure). Bottom figure shows distance from the illuminated water column in *Neobenedenia girellae* oncomiracidium.

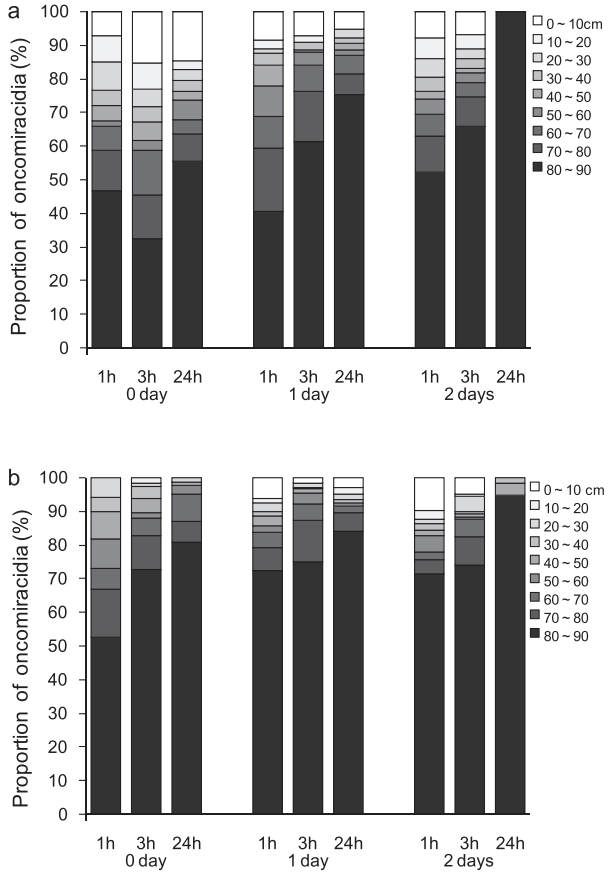

Fig. 6. Vertical distribution of *Heterobothrium okamotoi* oncomiracidium (0 day, 1 day and 2 days post hatch) after released from the top (a) and bottom (b) of a vertical column (900 mm in depth).

the bottom column tended to increase with time after release and time after hatch. Relatively high proportion of freshly hatched top-releasing larvae remained at the top column even after 24 h. However, all of 2 day old top-releasing larvae were found at the bottom 10 cm after 24 h. Interestingly, some of bottom-releasing lar-

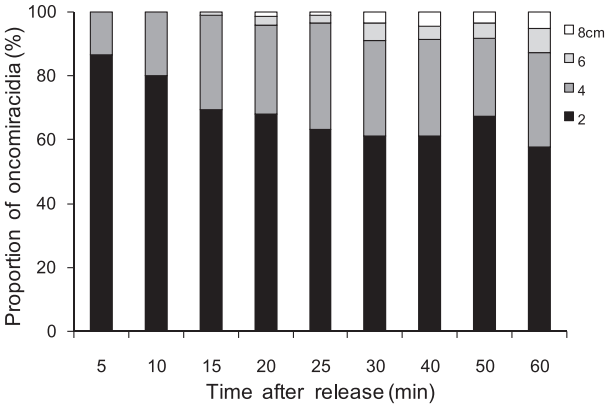

Fig. 7. Horizontal distribution of *Heterobothrium okamotoi* oncomiracidium in a glass dish. Proportions of larvae found in the four distance areas from the releasing point at each time point are shown.

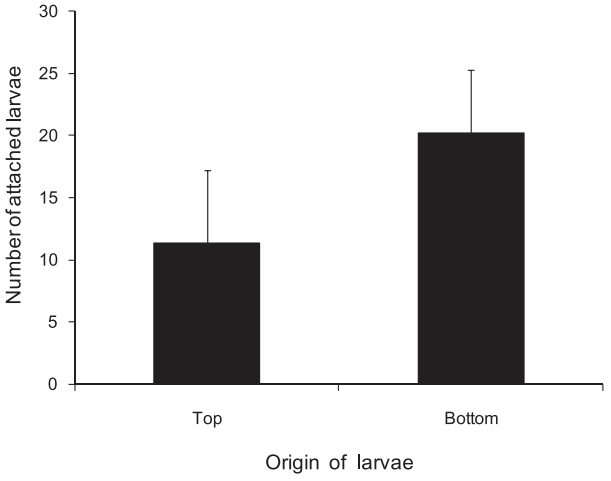

Fig. 8. Numbers of oncomiracidia collected from the top and bottom water column that were attached to fish gills and body surface. Data are expressed as mean + SD.

vae were found in the top 20 cm water columns which indicate that these larva moved upward.

Horizontal distributions of larval *H. okamotoi* in a glass dish were plotted in Fig. 7. The dispersion of the oncomiracidia was relatively slow. More than half of the larva remained within the 2 cm radius area even after 60 min. The maximum distance in which larvae were found was 8 cm from the releasing point.

Infection experiment

Nearly all (97.5%) of attached larval *H. okamotoi* were found on gills and the rest were found from the body surface. Higher numbers of bottom larvae (19.8 ± 4.7) attached to the host gill than the top larvae (11.2 ± 5.5) worms/host, Wilcoxon, $Z = -1.99$, $p = 0.046$). However, such difference was not statistically significant when larvae found on body surface were included (Wilcoxon, $Z = -1.89$, $p = 0.059$, Fig. 8). The mean

infection rates of bottom collected larvae (74.3 ± 18.6%) on the gills were more than 30% higher than those collected from the top column (41.9 ± 21.5%).

Discussion

The results of the present study demonstrated a clear behavioral difference among oncomiracidia of different monogenean species. Larval *H. okamotoi* spent relatively long time being stationary in the water whereas that of *H. heterocerca* continuously swam around. In other words, *H. heterocerca* was more active and had potential to cover larger distance in a given time than *H. okamotoi,* though their dispersion in the natural environment has to be studied. Such behavioral differences are not likely to be attributed to their size as the oncomiracidia of both species are around 200 μm in length (Ogawa and Egusa, 1981; Ogawa, 1998). Rather, these differences reflect the behavioral differences of their target hosts. Yellowtail, the host for *H. heterocerca,* is much more active and swims faster than tiger puffer, the host for *H. okamotoi*. In contrast to *H. okamotoi*, larval *N. girellae* showed strong positive phototaxis. Although *N. girellae* is capable of infecting a wide range of cultured fish species such as yellowtail, olive flounder and tiger puffer (Ogawa *et al*., 1995), its natural hosts are probably non-demersal fishes that swim close to the surface because the phototactic larvae should swim toward the light and distribute near the surface.

In aquaria, tiger puffer are often found at the bottom of the tank, burying part of their body into the substrate. The oncomiracidia of *H. okamotoi* did not show any phototaxis but had tendency to gradually accumulate in the lower water column. In addition, their horizontal dispersion rate was low, probably due to frequent circular movement. These results indicate that in the wild, hatched *H. okamotoi* gradually move downward toward the ocean floor where they prowl around in a random direction within a relatively small area.

The relatively higher infectivity of *H. okamotoi* oncomiracidia collected from the lower water column compared to those collected near the surface also suggests that the natural infection takes place closer to the ocean bottom. Because the top larvae were minority and apparently had a lower ability to infect fish, they might include abnormal individuals. More lipid droplets in the body (Ogawa, 1998) may have caused such maladaptive vertical distribution. Due to high host specificity of *H. okamotoi* (Ogawa, 1991), the oncomiracidia may have acquired adaptive behavior other than downward movement to locate the host. Hirazawa *et al*. (2003) reported that the *H. okamotoi* oncomiracidia uses body mucus pH as a cue to attach to the suitable fish host. To determine whether *H. okamotoi* possess sensory organs for particular chemical or uses other stimuli for finding the host requires further studies.

The motility of larval *H. okamotoi* showed gradual decline within a period of 3 days. Chigasaki *et al.* (2000) showed that the infectivity of *H. okamotoi* significantly reduced after 2 days. As the oncomiracidia do not feed, their activities entirely rely on stored energy (Bush *et al*., 2001). Although some of *H. okamotoi* larvae retained infectivity at 4 days post hatch, most of them must find the suitable host in narrow window period of only 2–3 days after hatch when they possess infectivity and motility, though the infection window may vary depending on the environmental temperature (Gannicot and Tinsley, 1998). Combining this with their low dispersion rate, *H. okamotoi* infection in the wild likely occurs within a relatively small area which tiger puffer inhabits in high density. The culturing conditions, thus, are ideal for the parasite to encounter the host. Because the larval *H. okamotoi* possess relatively low locomotive ability and short infection period, once the larvae are dispersed away from the culturing area, their chance of coming back and infect the cultured fish will be low. Therefore, use of water current by generating water flow or placing the net in the relatively high current area may be effective in reducing the infection. In addition, use of shallow floating net cages may reduce the chance of the parasite to attach to the host during their downward movement. However, it has to be noted that our data are based on laboratory experiments and observed behaviors and dispersal characteristics may differ under natural conditions.

In conclusion, our study indicates that the oncomiracidia of *H. okamotoi* tend to sink to the bottom and possess relatively low motility with no obvious moving pattern. Accumulation of such ethological information is important for deepening our understanding of disease-causing pathogens and for developing an ecological control measure that is safe for both humans and the environment.

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References

Bush, A. O., J. C. Fernandez, G. W. Esch and J. R. Seed (2001): Parasitism: The diversity and ecology of animal parasites. Cambridge University Press, Cambridge, UK. 554 p.

Chigasaki, M., K. Ogawa and H. Wakabayashi (2000): Standardized method for experimental infection of tiger puffer, *Takifugu rubripes* with oncomiracidia of *Heterobothrium*

okamotoi (Monogenea: Diclidophoridae) with some data on the oncomiracidial biology. *Fish Pathol*., **35**, 215–221.

Gannicott, A. M. and R. C. Tinsley (1998): Larval survival characteristics and behavior of the gill monogenean *Discocotyle sagittata*. *Parasitology*, **117**, 491–498.

Hirazawa, N., S. Oshima, T. Mitsuboshi and S. Yamashita (2003): Mucus pH of the tiger puffer *Takifugu rubripes* is an important factor for host identification by the monogenean *Heterobothrium okamotoi*. *Parasitology,* **127**, 225–230.

Ishida, M., F. Kawano, N. Umeda and N. Hirazawa (2007): Response of *Neobenedenia girellae* (Monogenea) oncomiracidia to brightness and black-and-white contrast. *Parasitology,* **134**, 1823–1830.

Naitoh, Y. and H. Kaneko (1973): Control of ciliary activities by adenosinetriphosphate and divalent cations in triton-extracted models of *Paramecium caudatum*. *J. Exp. Biol*., **58**, 657–676.

Ogawa, K. and S. Egusa (1981): Redescription of the development of *Heteraxine heterocerca* (Monogenea: Heteraxinidae) with a note on the relationship between the asymmetry and the site of attachment to the gill. *Bull. Jpn. Soc. Sci. Fish.,* **47**, 1–7.

Ogawa, K. (1991): Redescription of *Heterobothrium tetrodonis* (Monogenea: Diclidophoridae) and other related new species from puffers of the genus *Takifugu* (Teleostei: Tetraodontidae). *Jpn. J. Parasitol*., **40**, 388–396.

Ogawa, K., M. G. Bondad-Reantaso, M. Fukudome and H. Wakabayashi (1995): *Neobenedenia girellae* (Hargis, 1955) Yamaguti, 1963 (Monogenea: Capsalidae) from cultured marine fishes of Japan. *J. Parasitol.,* **81**, 223–227.

Ogawa, K. (1997): Copulation and egg production of the monogenean *Heterobothrium okamotoi*, a gill parasite of cultured tiger puffer (*Takifugu rubripes*). *Fish Pathol*., **32**, 219–223.

Ogawa, K. (1998): Egg hatching of the monogenean *Heterobothrium okamotoi*, a gill parasite of cultured tiger puffer (*Takifugu rubripes*), with a description of its oncomiracidium. *Fish Pathol*., **33**, 25–30.

Ogawa, K. (2002): Impacts of diclidophorid monogenean infections on fisheries in Japan. *Int. J. Parasitol*., **32**, 373-380.

Ogawa, K., N. Yamabata and T. Yoshinaga (2005): Egg-laying of the monogenean *Heterobothrium okamotoi* on experimentally infected tiger puffer *Takifugu rubripes*. *Fish Pathol*., **40**, 111–118.

Ohhashi, Y., T. Yoshinaga and K. Ogawa (2007): Involvement of host recognition and survivability in the host specificity of the monogenean parasite *Heterobothrium okamotoi*. *Int. J. Parasitol*., **37**, 53–60.

Yamabata, N., T. Yoshinaga and K. Ogawa (2004): Effects of water temperature on egg production and egg viability of the monogenean *Heterobothrium okamotoi* infecting tiger puffer *Takifugu rubripes*. *Fish Pathol*., **39**, 215–217.

Yoshinaga, T., M. Yasuzaki and K. Ogawa (2006): Fate of oncomiracidia of *Heterobothrium okamotoi* (Monogenea) attaching to the body surface of tiger puffer. *Fish Pathol*., **41**, 113–115.